Cold Shock Induces the Synthesis of Stress Proteins in Human Keratinocytes

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Heat shock proteins or stress proteins are synthesized when cells are exposed to a wide variety of physiologic stresses. The stress response is evolutionarily highly conserved, suggestive of an essential function(s) for the survival of organisms, protecting them from harmful trauma. Exposure to cold induces a stress response in organisms such as *Drosophila melanogaster* and *Sarcophaga crassipalpis* and this led us to determine whether or not cold shock responses occur in human skin after exposure to cold such as might occur during cryopreservation of tissues or cryosurgery. Biopsies taken from fresh human skin at chest surgery were exposed to 4, 15, 20, and 37°C (control) for 60 min and then allowed to incorporate ^{35}S-methionine at 37°C for up to 3 h. Proteins from the epidermis were extracted and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis. At 15°C and below there was increased synthesis of 90 and 72 kD proteins 2 h after shocking. The 72-kD protein was identified as a heat shock protein using a monoclonal antibody to HSP72 and it is proposed from electrophoretic evidence that the 90-kD protein is also a heat shock protein. Clearly, cold shock stimulates a stress response in human epidermis altering the spectrum of proteins expressed and inducing the synthesis of heat shock proteins. *J Invest Dermatol 101:196–199, 1993*

The heat shock or stress response may be induced in cells of any living organism from bacteria to humans by exposure to heat or by subjecting the cells to a number of other conditions, such as contact with heavy metals or treatment with ethanol, amino acid analogues, sulfydryl reagents, or even infection by certain viruses [1–3].

The response is characterized by a rapid increase in the rate of synthesis of a group of highly conserved proteins known as heat shock proteins (HSP), also called stress proteins. The wide variety of stressors that stimulate such a response are thought to share the common characteristics of either denaturing or damaging cellular proteins directly or causing cells to synthesize aberrant proteins [4]. The presence of denatured proteins is thought in some way to activate a "transcriptional heat shock factor" [5], although recent evidence suggests that in *Drosophila* heat shock factor activation is achieved by a heat-induced oligomerization [6]. Once the heat shock factor is activated, it binds to a short, highly conserved DNA sequence known as the heat shock element, and the induction of heat shock gene expression ensues [7,8].

The general role of HSP is a homeostatic one by which they protect the cell against harmful traumas and preserve cell viability, enabling a rapid return to normal cellular activities once the shock is over. The major mammalian HSP are classified into families on the basis of their molecular weights and sequence homologies, and include the 8-kD protein, ubiquitin, 20–28-kD proteins (small HSP), 60–65-kD proteins (HSP 60), 68–73-kD proteins (HSP 70), 83–90-kD proteins (HSP 90) [9].

The functions of the HSP90 and HSP70 families have been the most widely investigated. The proteins of the HSP70 family appear to bind to denatured and unfolded proteins [10] and prevent their further aggregation and precipitation. They also keep newly formed proteins destined for export unfolded and allow their translocation across cell membranes. In a similar way, they can behave as molecular chaperones mediating the proper folding and assembly of proteins. Proteins of the HSP90 family appear to mediate the action of some steroid hormones by masking the DNA binding site of hormone receptors until a hormone is positioned within the binding site [11]. Thus, in general, the protective role of the heat shock response is achieved by the ability of HSP to associate with other proteins in the cell and modify their function or fate.

It is possible that stress proteins play an important role in protecting keratinocytes in the skin from everyday environmental traumas such as extremes of temperature (e.g., when washing) or exposure to potentially toxic chemicals (e.g., household or environmental). Recently it has been reported that cold, another form of stress, may also induce the synthesis of stress or cold shock proteins in a variety of organisms such as *Drosophila* [12], the flesh fly *Sarcophaga crassipelpis* [13], *Escherichia coli* [14], and yeasts [15]. To our knowledge cold shock proteins have not been reported in humans. We thought it of interest to determine whether or not cold shock proteins are induced in human epidermis after exposure to cold such as might occur during cryosurgery, cryopreservation of tissue, or in cold injury such as frost bite. Therefore, we compared the effect of cold and heat on keratinocytes in human epidermis and in culture (squamous cell carcinoma 12F cells) and we report here that such cells do indeed show a typical stress response to these traumas.

MATERIALS AND METHODS

Effect of Cold and Heat Shock on Human Epidermal Biopsies Human skin biopsies (6 mm) were taken from chest skin at surgery and immediately exposed to cold or heat shock. Skin biopsies were shocked in glutamine-free modified Eagle's medium (MEM), either in the cold at 4, 15, or 20°C for 60 min or were heat shocked at 44°C for 60 min. Control biopsies were maintained at 37°C. The biopsies were then allowed to incorporate ^{35}S-methionine, 0.25 mCi/ml, in glutamine/methionine free MEM at 37°C for 2 h or at other times where indicated. After washing in ice-cold

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glutamine-free MEM, the epidermis was removed from the biopsies by keratotome (0.1 mm) and proteins were extracted in a 0.05 M Tris(hydroxymethyl)-aminomethane (Tris)/HCl buffer pH 7.2 containing 2% sodium dodecylsulfate (SDS) and 20 mM dithiothreitol, by sonication and heating at 80°C for 10 min, followed by centrifugation at 13,000 × *g* for 10 min.

Cold Shock of Cultured SCC Cells Human squamous cell carcinoma cells (SCC12F) were grown to near confluence in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, glutamine, hydrocortisone, and 1% Humalin S in 2-cm² plastic wells at 37°C in 5% CO_2. The cells were passaged, seeding at 10^5 cells per well and grown for 2–3 d to 5×10^5 cells per well. Single cell suspensions were prepared by detaching the keratinocytes from the substratum with 0.5% trypsin and 0.2% ethylenediamine tetraacetic acid (EDTA) followed by washing in DMEM. Squamous cell carcinoma cells were either maintained at 37°C for 1 h (control) or cold-shocked either by exposure to 4°C for 1 h or, alternatively, they were rapidly frozen in fetal calf serum containing 10% dimethylsulphoxide (added immediately before freezing) at −70°C for 2 h and then placed in liquid nitrogen overnight. Immediately prior to ^{35}S labeling cryopreserved cells were rapidly thawed at 37°C (3 min) and washed in methionine/glutamine-free MEM. Cold-shocked, cryopreserved, and control cells were then incubated in ^{35}S-methionine, 0.25 mCi/ml, in glutamine/methionine-free MEM at 37°C for 2 h. Proteins were extracted in 0.05 M Tris/HCl buffer, pH 7.2, with 2% SDS and 20 mM dithiothreitol as above.

Radioactivity Determination The amount of ^{35}S-methionine incorporation into protein was determined by precipitation with 10% trichloroacetic acid. Precipitates were collected under pressure filtration on Whatman Glass microfiber filters, washed thoroughly with ethanol and acetone, and dried, and the radioactivity was determined by liquid scintillation counting (Packard Emulsifier Safe scintillation fluid).

Electrophoresis and Autoradiography Extracted proteins, in the presence of 20% (v/v) glycerol and 0.005% (w/v) bromophenol blue, were analyzed by SDS-polyacrylamide slab gel electrophoresis on 7.5–17.5% gradient (acrylamide/bisacrylamide ratio 75:2) with a 4% (w/v) stacking gel, in a discontinuous Tris/glycine buffer system. Equal amounts of trichloroacetic acid precipitable counts were loaded for each sample on a gel. Gels were stained with 0.1% Coomassie brilliant blue R and dried. The incorporation of ^{35}S-methionine into proteins was visualized by autoradiography using Fuji FX x-ray film at −70°C and quantified by scanning densitometry of the autoradiograms using an LKB Ultrascan laser densitometer. Polypeptide peak areas (mm²) were normalized by proportional adjustment after standardization of the total peak areas for each lane of a gel. Keratins 57/59 kD (K10/K5) or a 42-kD protein were used as the invariant control.

Immunoblotting Gels were subjected to immunoblotting [16] using a monoclonal antibody C92, against a shared epitope of the inducible and constitutively expressed mammalian HSP 72/73. The antibody was used at a dilution of 1:1000. The blots were visualized by staining with a rabbit anti-mouse horseradish–peroxidase-conjugated antibody (DAKO) at 1:20 dilution and using diaminobenzidine as the substrate. (The monoclonal antibody was a kind gift by Dr. W.J. Welch, Lung Biology Center, San Francisco, California.)

RESULTS

Induction of Stress Proteins by Cold in Human Epidermis The effect of cold on the protein expression of human epidermal keratinocytes *in vitro* at temperatures of 4, 15, and 20°C is shown in the autoradiogram in Fig 1. Cold-induced changes were identified by comparison with the control incubation at 37°C. Quantification by laser densitometry showed that the greatest increases in labeling occurred in the 90-kD and 72-kD proteins. There was an approximately twofold increase in the 90-kD band at all shocking temperatures as compared with the control. A similar change was observed in the amount of 72-kD protein at 4°C, with a smaller increase at 15°C, but no change at 20°C (Table I).

The time course for the synthesis of these cold shock proteins after treatment at 4°C was determined by analysis at 30, 60, 90, 120, and 180 min. A Western blot of this time course gel using the monoclonal antibody (C92) to the inducible HSP72 and the constitutive HSP73 is shown in Fig 2. The antibody identified the constitutive protein and also bound to the 72-kD cold-shock protein, showing that it was also a heat-shock protein. The data from the blot experiment suggest that after cold treatment the inducible HSP72 levels increased to a maximum at 90 min and that levels were returning to normal by 180 min.

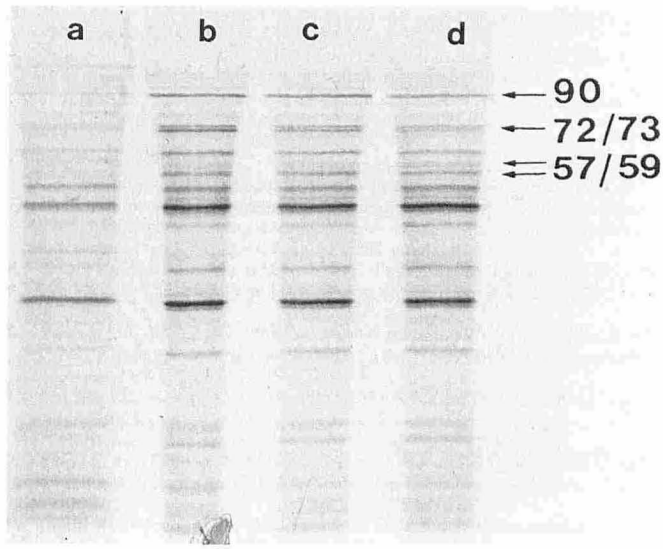

Figure 1. Autoradiogram of sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of proteins extracted from control and cold-treated epidermal keratinocytes. Skin biopsies were incubated at 37°C for 2 h in ^{32}S-methionine after treatment at 37°C for 1 h *(lane a)*, cold shocked at 4°C *(lane b)*, 15°C *(lane c)*, or 20°C *(lane d)* for 1 h. Proteins were extracted from the epidermis and equal amounts of trichloroacetic acid precipitable radioactivity were analyzed by SDS-PAGE (7.5–17.5%) and autoradiography. The positions of the stress proteins 90 and 72/73 are indicated as well as the invariant control 57/59. Mr are given in kD.

Induction of Stress Proteins by Cold in Cultured SCC Cells An investigation of the effect of cold on SCC12 cells (Fig 3) revealed that there was very little difference in the proteins synthesized by cells after cryopreservation from the proteins synthesized by cells that had not been stressed, but maintained for 1 h at 37°C. Cold shock at 4°C for 1 h induced a greater change in the synthesis of the 72-kD protein. Scanning densitometry showed that there was a 50% increase in the 72-kD protein after cryopreservation and a 100% increase of this protein after cold shock treatment.

Comparison of the Effect of Cold and Heat on Human Epidermis The effect of cold at 4°C was compared to the response induced by heat at 44°C (Table II). Heat shock produced the greatest changes in epidermal proteins. Extra stress proteins with Mr 112 kD and 41 kD were induced, but the most obvious difference was the increased labeling of the 90 and 72 HSP. The synthesis of the 90-kD and 72-kD HSP were quantified in three different skin samples after cold and heat shock. For the 90-kD protein, after heat shock at 44°C, there was a 2.6-times increase in level compared

Table I. Effect of Cold Shock on Stress Protein Synthesis in Epidermal Keratinocytes[a]

Stress Protein	Normalized Polypeptide Peak Areas (mm²)[b]			
	37°C	4°C	15°C	20°C
90 kD	0.25	0.54	0.59	0.50
72/73 kD	0.54	0.93	0.71	0.51
Invariant control 57/59 kD	0.92	0.85	0.91	0.89

[a] Skin biopsies were kept at 37°C for 1 h (control) or cold shocked at 4, 15, or 20°C for 1 h and then returned to 37°C and labeled with ^{35}S-methionine for 2 h. Proteins were extracted from the epidermis and equal amounts of trichloroacetic acid precipitable radioactivity were analyzed by SDS PAGE, visualized by autoradiography, and quantified by scanning laser densitometry.

[b] Densitometry data was normalized by standardization of the total peak areas for each lane of the gel and the peak area of individual proteins in each track was then altered proportionately.

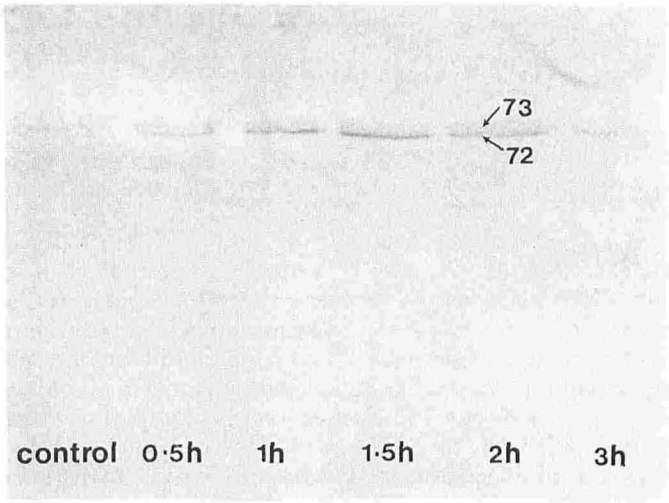

Figure 2. Immunoblot of the time-course of biosynthesis of HSP 72 by epidermal keratinocytes. Skin biopsies were cold shocked at 4°C for 1 h. At various times, 0.5 h, 1 h, 1.5 h, 2 h, and 3 h, during recovery at 37°C from the cold shock, proteins were extracted from the epidermis, analyzed by SDS-PAGE (7.5–17.5%), transferred to nitrocellulose paper, and probed with the monoclonal antibody "C92" to the HSP 72/73 (dilution 1:1000).

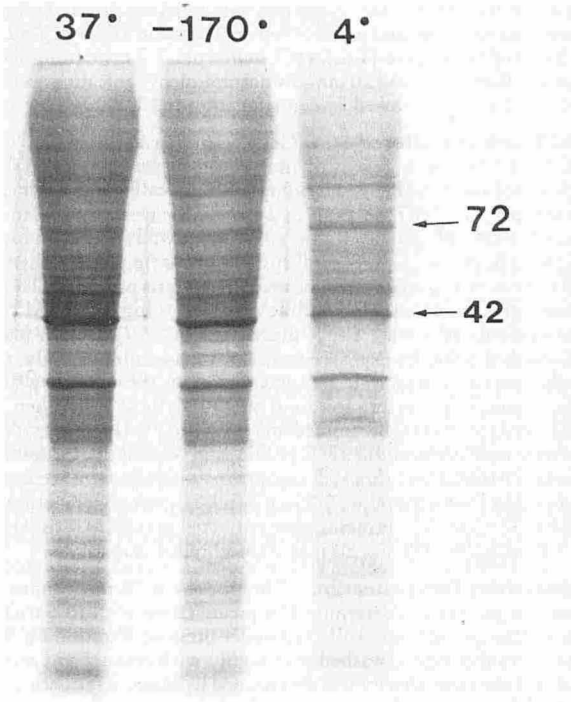

Figure 3. Autoradiogram showing the effect of cold shock on stress protein synthesis in SCC12F cells, which were either cold shocked at 4°C for 1 h, cryopreserved overnight in liquid N^2 (−170°C), or kept at 37°C for 1 h (control). Cells were subsequently labeled with ^{35}S-methionine at 37°C for 2 h and proteins detected by SDS-PAGE and autoradiography. Cryopreserved cells were thawed rapidly (37°C/3 min) immediately prior to ^{35}S-methionine labeling at 37°C for 2 h. The position of the 72-kD stress protein is indicated, as well as the invariant control 42. Mr are given in kD.

with the control, whereas after cold shock at 4°C there was a 1.7-times increase. For the 72-kD protein, after heat shock at 44°C there was a 4.3-times increase in level, whereas after cold shock at 4°C there was a twofold increase.

DISCUSSION

Sudden, relatively short, low-temperature exposure consistently and specifically altered the expression of proteins synthesized by keratinocytes, either normal ones from human epidermis or SCC12F cells in culture. In epidermal keratinocytes, *in situ* there was increased synthesis of two proteins of Mr 90 kD and 72 kD, whereas in cultured keratinocytes only the amount of the 72-kD protein was increased.

The cold shock/stress protein of Mr 72 kD was shown to be identical with heat shock protein 72 kD by using a specific antibody. It seems almost certain that the 90-kD protein was also a stress protein and thus a common characteristic of both heat- and cold-stressed epidermis is the increased synthesis of HSP72 and HSP90. However, the magnitude of the response varied with the nature of the stress and in general the changes produced by heat shock of a few degrees were far greater than those produced by cold shock. One question that remains is whether or not these heat shock proteins were induced as a result of the metabolic stress of proteins being denatured by cold shock, or their induction was due to the keratinocytes' response to the temperature shift from 4°C to 37°C, that is, effectively a heat shock after cooling. The latter seems less probable as the rapid temperature change to the normal physiologic one would seem to be unlikely to promote the denaturation of proteins and the resultant initiation of the stress response. In contrast, the slower change to lower temperatures and maintenance at the low temperature might well be more conducive to protein denaturation and the initiation of a stress response. Also, heat-shock proteins are clearly synthesized during the actual heat shocking, but cold shock/heat shock proteins were not detected until at least 30 min after cold treatment. This may mean that the induction pathways are not the same for a heat- or cold-shock response. However, it needs to be remembered that all the cellular processes are slowed down by the drop in temperature. Even the chemical steps by which the stress response is mediated will be slowed.

Other evidence from experiments with *Drosophila* and flesh fly [13,17] suggests that HSP are synthesized during recovery from cold shock. In both these insects, injuries incurred by brief exposure to high or low temperatures were prevented by previous exposure to less severe temperatures. Indeed, a brief exposure to a high temperature provided protection against cold-shock injury. It was reported that in yeast cells [18] the induction of HSP synthesis by pre-incubation at heat-shock temperatures conferred protection against subsequent cryoinjury by freezing in liquid nitrogen. Heat shock proteins are thought to offer protection by macromolecular stabilization and by increasing hydrophobic interactions, i.e., they may behave as

Table II. Effect of Cold or Heat on the Synthesis of 72/73 kD and 90-kD Polypeptides in Epidermal Keratinocytes[a]

Stress Protein	Skin Sample	Normalized Polypeptide Peak Areas (mm^2)[b] 37°C	4°C	44°C
90 kD	1	0.21	0.48	0.62
	2	0.15	0.23	0.38
	3	0.25	0.35	0.61
72/73 kD	1	0.45	0.96	2.27
	2	0.30	0.62	1.28
	3	0.50	0.84	1.78
Invariant Control 57/59 kD	1	1.29	1.20	1.20
	2	0.84	0.86	0.84
	3	1.00	1.03	1.00

[a] Skin biopsies were kept at 37°C for 1 h (control), cold shocked at 4°C for 1 h or heat shocked at 44°C for 1 h and then returned to 37°C and labeled with ^{35}S-methionine for 2 h. Proteins were extracted from the epidermis and equal amounts of trichloroacetic acid precipitable radioactivity were analyzed by SDS PAGE and autoradiography and quantified by scanning laser densitometry.

[b] Densitometry data was normalized by standardization of the total peak areas for each lane of the gel and the peak area of individual proteins in each track was then altered proportionately.

cryoprotectants. Thus, HSP72 and HSP90 synthesized during the recovery of keratinocytes from cold shock could play a protective role if the epidermis was again presented with a low-temperature challenge. Such implications could be of relevance in the cryopreservation of skin or other tissues for future surgical use. For example, the induction of HSP synthesis by pre-incubation of skin at high and low temperatures might confer tolerance. Certainly, such protection must occur in keratinocytes in skin *in vivo* because many temperature changes are experienced in everyday life.

It has been suggested that the consistent presence of HSP72 in human keratinocytes after exposure to heat or chemical shock makes it a useful indicator of cellular stress [19]. The small increase in HSP72 in SCC 12F cells after cryopreservation in the presence of fetal calf serum and dimethylsulphoxide in liquid nitrogen and the similarity in protein synthesis with cells maintained at 37°C, could suggest that the cells had been adequately protected during freezing and thawing with little cell damage. The presence of HSP72 might, therefore, be a useful marker of cell injury during the cryopreservation of tissues.

Finally, the roles played by stress proteins in the protection and recovery of keratinocytes from cold are not yet understood. It is likely that HSP72 will be involved in the salvaging of denatured proteins either by solubilizing them and facilitating their refolding or by chaperoning them to a degradative system, whereas other cold-induced proteins may play an important part in the reinitiation of macromolecular synthesis.

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